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Modulation of PPAR-Gamma Signaling in Prostatic Carcinogenesis

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**13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)**

The long term **objective** of this work is to elucidate metabolic pathways which can be used to reduce the need for radical surgery in patients at high risk for prostate cancer or with early stage disease. The **hypothesis** to be tested is that alterations to lipoxygenase (LOX) and cyclooxygenase (COX) activity in early prostate cancer represent distinct druggable pathways which can be treated in conjunction with the PPARγ signaling pathway to slow or prevent the development and progression of prostate cancer. In the first year of funding, we have generated many of the viral vectors (PPARγ siRNAs, COX and LOX shRNA and overexpression) needed to perform the experiments in the bulk of the proposal. We have completed the majority of the experiments proposed in specific aim 1 relating to the knockdown of PPARγ in human prostatic epithelial cells and have confirmed that these cells behave as predicted using in vivo models. These experiments are currently undergoing the final stages of analysis. We have started generation of cells with altered COX and LOX expression for the experiments proposed in specific aim 2. Work for specific aim 3 has not yet begun. Major findings from the first year of work are that human prostatic epithelial cells, as expected, respond to loss of PPARγ signaling in the same way as mouse cells.

**14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)**

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Annual Report

PCRP Idea Development Award

W81XWH-07-1-0479

Modulation of PPAR-Gamma Signaling in Prostatic Carcinogenesis

**P.I. Simon W. Hayward, PhD**

## Introduction

This project examines the relationship between PPARγ and carcinogenesis. PPARγ sits at a critical juncture in cellular differentiation and metabolism being involved in both differentiation and in the regulation of stress responses mediated through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways of fatty acid metabolism. The basis for this project was the observation that in human prostate cancer there is an early loss of enzymes responsible for the production of the putative endogenous ligands for PPARγ, presumed to result in a decrease in receptor function. We have found that loss of PPARγ function can result in the generation of premalignant prostatic lesions in mice (paper in submission). We have also shown that there is an associated upregulation of COX pathways which would generate increases in prostaglandin production and oxidative stress, which could underlie such a pathology. This project sets out to examine interactions between the PPARγ, COX and LOX pathways and their role in carcinogenesis. We are using predominantly tissue recombination models involving human prostatic epithelial cells. The use of human cells is important in that there are significant differences between the fatty acid metabolic pathways between humans and mice.

## Body

Status of progress in relation to the original SOW is summarized below:

Task 1. Examine the in vivo consequences of suppression of PPARγ signaling in human prostatic epithelium.

Generate and test PPRE-luciferase reporter construct (months 1-3) **Ongoing. This construct currently works for mouse cells in our hands and is being optimized for human cells.**

Generate and test viral vectors carrying shRNA targeting human PPARγ (months 1-4) **Completed**

Grow up and infect hPrE and hPrEshp16 cells using PPARγ shRNA and PPRE-luc viral particles. Select infected cells (months 4-8). **Completed shRNA phase – some modifications noted below.**

Validate function of viral constructs in cells (Western blot and luciferase assay – qRT-PCR can also be used for the PPRE-luc is PPARγ suppression is unexpectedly efficient) (months 5-9) **Western blot completed, RT-PCR confirmation pending.**

Generate and graft tissue recombinants using infected epithelial cells and NPF (or rUGM controls) (months 6-10) **Completed for rUGM, in progress for NPF.**

Harvest recombinants, process for biochemical and immunohistochemical analysis (months 10-17) **In progress, the majority of grafts have been harvested and section immunostaining is ongoing.**

Histopathologic assessment of slides (months 15-20). **Pending completion of immunohistochemistry, preliminary findings presented below.**

Generation and analysis of proliferative and apoptotic indices (months 15-20) **Not yet started.**

Summation of data set and manuscript preparation (months 20-24) **Not yet started.**

Task 2. Examine the in vitro and in vivo consequences of overexpression of cyclooxygenase –1 or –2 or 15-lipoxygenase-1 in human prostatic epithelium.

Generate and test viral vectors carrying expression constructs for COX-1, COX-2 and 15-LOX-1 (months 2-5). **COX-2 and 15-LOX-2 overexpression and knockdown completed. COX-1 and 15-LOX-1 pending.**

Grow up and infect hPrE and hPrEshp16 cells using PPRE-luc viral particles in combination with COX-1, -2 or 15-LOX-1 overexpression viral vectors. Select infected cells (months 5-9). **COX-2 and 15-LOX-2 overexpression and knockdown completed. COX-1 and 15-LOX-1 pending.**

Validate function of viral constructs in cells (Western blot and luciferase assay) (months 5-10) **COX2 and 15-LOX2 overexpression and knockdown completed. COX1 and 15-LOX-1 pending.**

Generate and graft tissue recombinants using infected epithelial cells and NPF (or rUGM controls) (months 6-10) **COX-2 and 15-LOX-2 overexpression and knockdown completed. COX-1 and 15-LOX-1 pending.**

Harvest recombinants, process for biochemical and immunohistochemical analysis (months 10-17) **COX-2 and 15-LOX-2 overexpression and knockdown completed. COX-1 and 15-LOX-1 pending.**

Histopathologic assessment of slides (months 15-20) **Ongoing.**

Generation and analysis of proliferative and apoptotic indices (months 15-20) **Not yet started.**

Summation of data set and manuscript preparation (months 20-24) **Not yet started.**

Task 3. Examine protective effects of PPARγ agonists and/or COX/LOX inhibitors against the neogenesis of PIN or progression of prostate cancer.

Generate and graft tissue recombinants of hPrEshp16 cells and rat UGM (or NPF) to SCID mouse hosts. Hosts divided into groups and fed for 7 days prior to grafting, and then throughout experiment on control chow, Avandia chow, Celecoxib chow and Avandia/Celecoxib combination chow. Additional combinations to be added depending upon the results of specific aim 2 (months 10-28) **Not yet started.**

Xenograft human prostate tumor tissue derived from Gleason grade 2+3 or 3+3 tumors to sets of SCID mice fed for 7 days prior to grafting, and then throughout experiment on control chow, Avandia chow, Celecoxib chow and Avandia/Celecoxib combination chow. Additional combinations to be added depending upon the results of specific aim 2 (months 10-28) **Not yet started, considering technical modification based upon experience in the period since project submission – discussed below.**

Harvest grafts starting three months post grafting, process for further analysis (months 13-31) **Not yet started.**

Histopathologic assessment of slides (months 15-34) **Not yet started.**

Generation and analysis of proliferative and apoptotic indices (months 15-34) **Not yet started.**

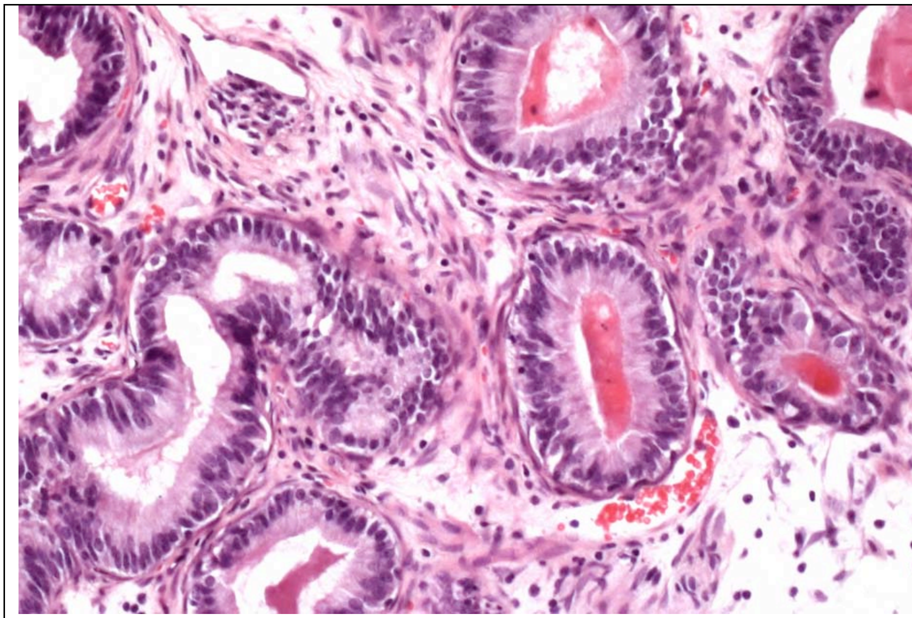
Summation of data set and manuscript preparation (months 34-36) **Not yet started.**

**Summary of Activity**

We have been pursuing the project as outlined in relation to the statement of work, as noted above. In general, the work is proceeding as planned and on time. The mouse work, which was the basis for this project, although not strictly a part of it, is completed and has been submitted for publication. Confirmation that the same premalignant changes seen in murine cells also occur in human epithelial cells has been generated using shRNA/siRNA knockdown of PPARγ-1/-2 in human prostatic epithelial cell lines followed by tissue recombination with prostate-inductive rat urogenital sinus mesenchyme (rUGM).

Since the submission of this proposal we have generated new human prostatic epithelial cell lines (figure 1) which better recapitulate human prostatic biology than those that were available at the time of submission. These have been successfully tested to examine their ability to form normal and benign prostatic structures in recombination with rat UGM (see figure 1) and have been shown to recapitulate the major key differentiation features of prostatic tissue, notably histopathologic organization and the expression of key markers of differentiation, notably appropriate basal and luminal cell phenotypes and

keratin expression profiles and the expression of important markers of prostatic differentiation such as androgen receptor and prostate specific antigen (PSA). Since these lines are well defined and allow better comparisons between experiments we will use these, rather than the primary cultures originally proposed for the bulk of the PPARγ, COX and LOX pathway manipulation studies.



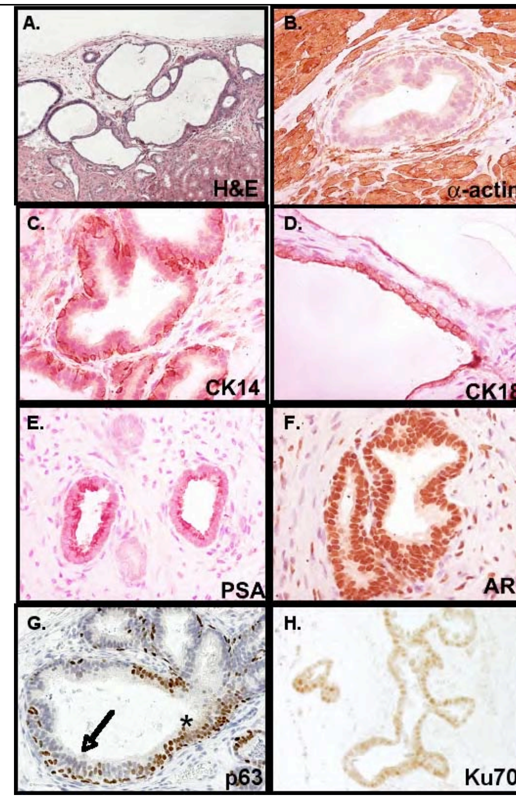



**Figure 1. HPrE cells.** We have developed two benign human prostatic epithelial cell lines (NHPrE1 and BHPrE1) for use in both *in vitro* and *in vivo* studies. These cells do not form tumors *in vivo* but retain expression of appropriate cytokeratins, and androgen receptor (AR), they are also able to express key prostate-specific markers such as PSA when recombined with an appropriate inductive mesenchyme (here rat urogenital sinus mesenchyme).

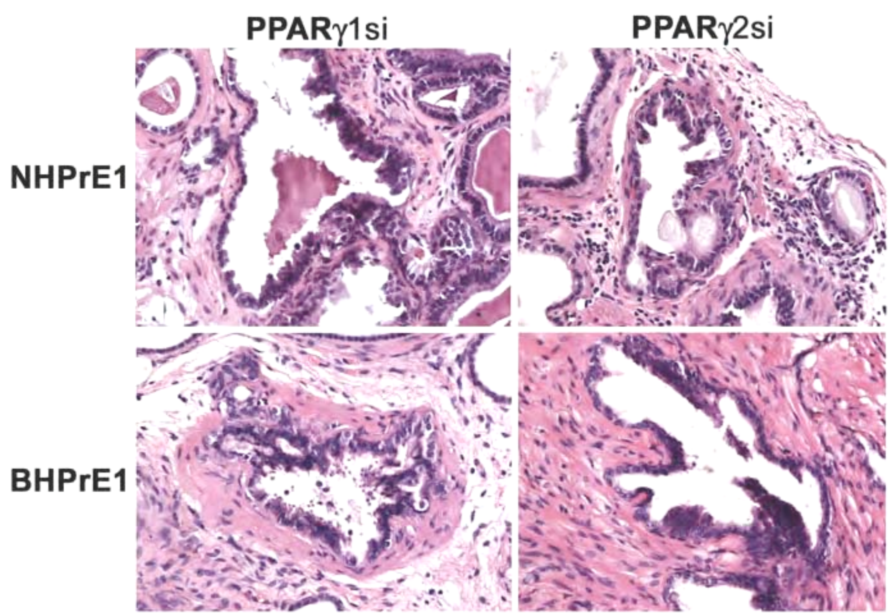



**Figure 2. PPARγ suppression.** Consequences of PPARγ suppression in NHPrE1 and BHPrE1 cells in tissue recombinants using rUGM. Suppression of either PPARγ1 or -γ2 resulted in the formation of PIN-like lesions as previously seen in mouse epithelial cells with the same genetic changes.

PPARγ expression (either PPARγ1 or γ2) was suppressed in NHPrE1 and BHPrE1 epithelial cell lines (task 2) and tissue recombinants generated using rUGM as an inductive mesenchyme. As shown in figure 2, this resulted in a pre-neoplastic PIN like phenotype. This result confirmed that human prostatic epithelial cells responded to loss of PPARγ in a manner which was phenotypically similar to previous results in mouse cells and consistent with the central concept of this proposal; that loss of this signaling pathway would predispose cells to malignant progression.

We initially proposed to use epithelial cells in which p16 was knocked down as a second genetic insult. We have also been developing the capacity to knock down expression of PTEN, which is commonly lost in human prostate cancer and propose to use this as an additional genetic insult to test in combination with loss of PPARγ signaling.

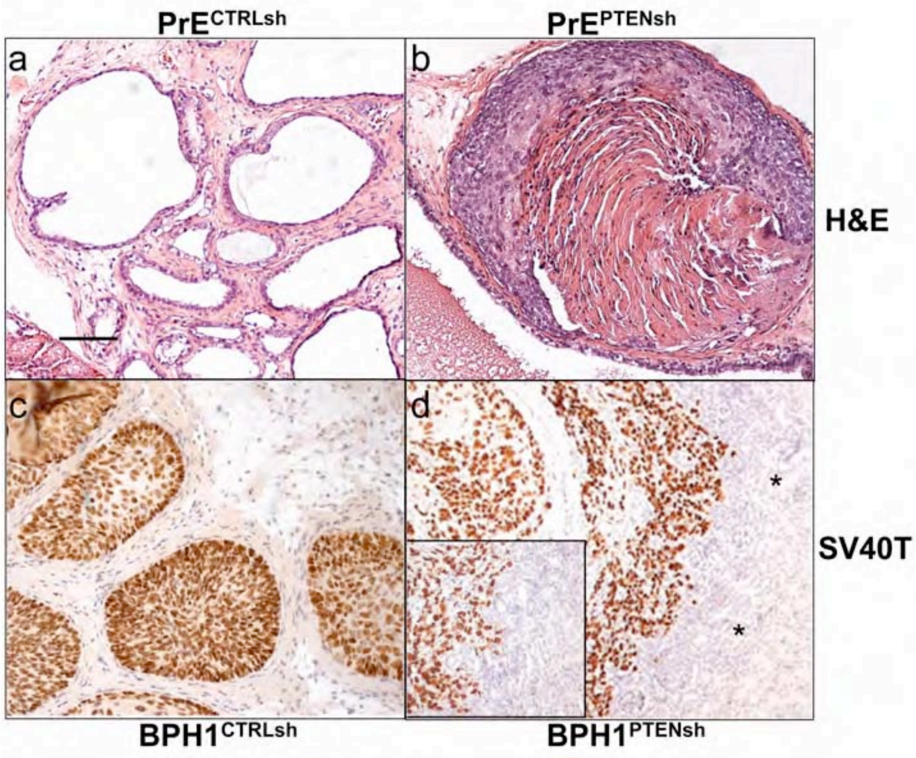



**Figure 3. Effects of PTEN Suppression.** PTEN suppression in normal and initiated prostate epithelia causes cellular piling and invasion, respectively.

Knockdown of PTEN in human primary cultures of epithelium (PrE) resulted in PIN lesions, while knockdown in the BPH-1 line (immortalized with SV40 large T antigen) resulted in invasive cancer (figure 3), showing the potential of this lesion to act as a potentiating insult promoting prostatic carcinogenesis. This suggests that this will be a useful model to pursue which is likely more clinically relevant than the p16 suppression which was initially proposed. Dependent upon a comparison of the results between the two cell types (shPTEN vs shp16) which are now ongoing, we would propose to move studies more towards PTEN suppression and away from p16 suppression.

As an additional experimental twist we have also developed (in relation to another study) tetracycline regulatable constructs. This allows us to regulate PTEN expression in vivo should such a manipulation be considered desirable. The use of this construct in vitro is illustrated in figure 4.

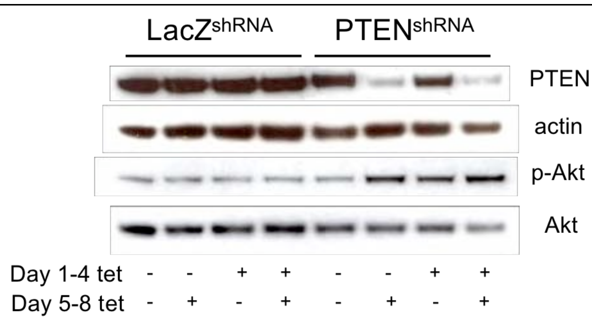



**Figure 4.** The reversible decrease of PTEN protein in BHPrE1 prostate epithelial cells by tetracycline-induced shRNA production. Tetracycline treatment was performed in two, 4-day stages. Decreased PTEN is observed within 4 days (lane 6). The reversibility of protein reduction can be seen when tetracycline was removed from the culture media for an additional 4 days (lane 7). Maximal PTEN reduction is seen at 8 days treatment (lane 8). A concomitant modulation of Akt phosphorylation is also observed, indicating a functional impact on signal transduction.

In task 2 we have started studies in which the expression of lipoxygenase and cyclooxygenase is regulated (expression or suppression). Early studies in which 15-Lox-2 is suppressed resulted in smaller cell in vitro with increased proliferation rates. Tissue recombinants incorporating these cells are being tested in vivo at the time of writing but no data are presently available.

Human prostate cancer xenografts (task 3): Over the last few years there has been a move toward the use of robotic laparoscopic surgery for radical prostatectomy. This has had the unfortunate side effect of degrading the quality of tissues available for research since these tissues spend considerably more time in the patient at body temperature with no blood supply than those from more traditional open resections. Since there is still a number of procedures performed which use the traditional approach, we are coordinating with the VICC Tissue Acquisition Core to ensure, before we start the work in the second part of task 3, that the tissue which we are receiving is screened so that robotically resected samples are excluded from the analysis.

**Key Research Accomplishments**

- Completed characterization of mice with conditional knockout of PPARγ in the prostate. Manuscript describing this work currently in submission.
- Generated knockdown of PPARγ-1/-2 in human prostatic epithelial cells. Demonstrated that in tissue recombination models these undergo similar profiles of phenotypic changes to those seen in mouse prostate in which expression of this gene is suppressed.

- Generated cells in which both PPARγ-1/-2 and PTEN expression were suppressed.
- Generated human prostatic epithelial cells overexpressing COX-2 and 15-LOX-2, also cells with these functions knocked down with siRNA or shRNA.
- Generated tissue recombinants using rUGM and human prostatic epithelial cells in which COX-2 or 15-LOX-2 were either overexpressed or suppressed.

**Reportable Outcomes**

None

**Conclusions**

This work is proceeding along the lines proposed. There have been minor modifications to the cell lines used which account for technical advances made after the proposal was submitted. We have included some additional genetic changes in the analysis in combination with alterations in PPARγ and the COX and LOX pathways, notably suppression of PTEN which result in premalignant lesions in these cells, and importantly, in combination with loss of PPARγ reflects a more accurate representation of the changes which are known to occur early in human prostate carcinogenesis, increasing the clinical relevance of the study.